GeneSearch™

GeneSearch™ Breast Lymph Node (BLN) Test Kit REF **2900004**

Σ 30 Reaction Kit

Instructions for Use

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The GeneSearch™ Breast Lymph Node (BLN) Assay may be used in conjunction with sentinel lymph node biopsy for a patient who has been counseled on use of this test and has been informed of its performance. False positive results may be associated with increased morbidity. False negative and inconclusive test results may be associated with delayed axillary node dissection. Clinical studies so far are inconclusive about a benefit from treatment based on findings of breast cancer micrometastases in sentinel lymph nodes.

Refer to the patients' and physicians' brochures, which are available on the Veridex Web Site or by contacting Veridex Customer Technical Services.

INTENDED USE

For *in vitro* diagnostic use only.

The GeneSearch™ Breast Lymph Node (BLN) Assay is a qualitative, in vitro diagnostic test for the rapid detection of greater than 0.2 mm metastases in nodal tissue removed from sentinel lymph node biopsies of breast cancer patients. Results from the assay can be used to guide the intra-operative or post-operative decision to remove additional lymph nodes.

Post-operative histological evaluation of permanent sections of the tissue specimen, in accordance with usual diagnostic practice and using the Veridex lymph node cutting scheme, is required.

SUMMARY AND EXPLANATION

The GeneSearch™ BLN Assay is a real time reverse transcriptase polymerase chain reaction (RT-PCR) assay that detects the presence of breast tumor cell metastasis in lymph nodes through the detection of gene expression markers Mammaglobin (MG) and Cytokeratin 19 (CK19), that are present in higher levels in breast tissue, but not in nodal tissue (cell type specific messenger RNA). This assay employs real time RT-PCR utilizing the Cepheid SmartCycler® system to generate expression data for the genes of interest. The expression results are then applied against predetermined criteria to provide a qualitative result. Results may be Positive or Negative or Invalid. Results of the assay correlate with detection of metastasis by paraffin-embedded (permanent section) hematoxylin and eosin (H&E) histology. (See the CLINICAL PERFORMANCE section later in these Instructions for Use.)

PRINCIPLES OF THE PROCEDURE

Real Time RT-PCR Reaction

The GeneSearch™ BLN Assay qualitatively detects the expression of two genes, Mammaglobin (MG) and Cytokeratin 19 (CK19). The specificity of MG for breast tissue and its usefulness in the detection of metastatic disease in lymph nodes, blood, and marrow has been reported[1, 2]. CK19 is an epithelial cell marker that has been frequently associated with

breast cancer in lymph nodes, bone marrow, and blood, and is expressed at levels a million-fold higher in cells associated with cancer compared to normal cells.[3] Using tissue RNA from all available sentinel lymph nodes of 254 subjects, the combination of MG and CK19 was reported to be optimal for the detection of breast cancer metastasis to lymph nodes.[4]

In order to maximize the uniformity of distribution of tissue sampling, lymph nodes are divided into sections and alternating sections are combined and processed using the GeneSearch™ BLN Assay. The remaining sections are used for routine histologic evaluation. Each lymph node is individually processed. Using the GeneSearch™ RNA Sample Preparation Kit, the nodal tissue is homogenized to release RNA molecules. The RNA is purified from the tissue homogenate and RT-PCR is performed on the RNA specimen.

The real time RT-PCR reaction is performed in a homogeneous, one-step, fully contained reaction. Three gene markers (MG, CK19 and an internal control gene [IC; porphobilinogen deaminase, PBGD]) are included in this reaction. A complementary DNA (cDNA) strand is produced from messenger RNA (mRNA) using the reverse transcriptase function of a thermostable DNA polymerase. The reaction containing marker-specific DNA primers and probes, deoxyribonucleoside triphosphates [dNTPs], and DNA polymerase in a buffer is heated to activate the DNA polymerase and then cooled to allow specific annealing of the target-specific reverse (antisense) primers to the target mRNAs. The annealed primers are extended by the DNA polymerase in the presence of excess dNTPs to form cDNA strands.

Following production of cDNAs, the reaction mixture containing the cDNA:RNA hybrid is again heated to denature the strands. The reaction mixture is cooled, allowing the target-specific forward (sense) primers to anneal, and allowing the DNA-dependent DNA polymerase activity to extend the sense strand through to the reverse primer regions. This amplification process results in double-stranded DNA sequences called amplicons. Subsequent cycles of denaturation and annealing/extension exponentially increase the amounts of these amplicons, which are subsequently detected utilizing sequence-specific DNA probes.

Detection of Gene Markers

Production of target amplicon is detected using a probe, as part of the RT-PCR mixture, that contains a DNA sequence specific for part of the target amplicon. This probe is linked to a fluorescent molecule and a molecule that quenches fluorescence. The probe initially anneals to the target sequence and then is cleaved by the exonuclease activity of the DNA polymerase as extension from the primer proceeds past the probe region. As a result of this cleavage, the fluorescent molecule is separated from the quencher, leading to an increase in fluorescence. By measuring fluorescence, the presence of target amplicon can be detected.

Each gene marker is detected using fluorescent molecules with different excitation and emission wavelengths. Fluorescence for each of the gene markers is measured following each temperature cycle. Amplification of the gene markers is detected through increased fluorescence due to release of the fluorophore from the proximity of the quencher. The Ct value is determined when the fluorescent signal exceeds a pre-defined threshold limit. If the external controls are valid, then the Ct value for each gene marker in the patient sample is compared to marker-specific Ct cutoff values. If the Ct value for either or both gene markers is less than the cutoff value then the sample is determined to be positive. All amplification,

detection of fluorescence, and the interpretation of the signals are done automatically by the SmartCycler® instrument.

MATERIALS PROVIDED

GeneSearch™ Breast Lymph Node (BLN) Test Kit – Part # 2900004

- Instructions for Use
- 0.3 mL Master Mix (White cap): Contains Tris buffer, 0.05% Bovine Serum Albumin, 0.08% Proclin 300, < 0.01% primers and probes
- 0.3 mL Enzyme Mix (Yellow cap): Contains Tris buffer, 0.08% Proclin 300, < 0.02% DNA polymerase (enzyme), and a proprietary stabilizing agent
- 0.05 mL Negative Control (Purple cap): Contains Tris buffer, < 0.001% plasmid DNA, 0.08% Proclin 300
- 0.05 mL Positive Control (Green cap): Contains Tris buffer, < 0.001% plasmid DNA, 0.08% Proclin 300

GeneSearch™ RNA Sample Preparation Kit – Part # 2900005

- 100 mL Homogenization Buffer: Contains ≥ 25% guanidine thiocyanate
- 8 mL Wash Buffer 1: Contains <10% guanidine thiocyanate, 10% ethanol
- 2 mL Wash Buffer 2: Contains proprietary compound mixture, 0.09% sodium azide, pH 7.5
- 1.9 mL RNase-free water
- 10 RNA Spin Columns

GeneSearch™ BLN Protocol Software CD, IVD – Part # 2900006

- GeneSearch™ BLN Protocol Software Release and Install Notes

MATERIALS REQUIRED BUT NOT PROVIDED

Laboratory Reagents

- β-Mercaptoethanol, molecular biology grade, 14.3 M
- 200-proof (absolute) ethanol, molecular biology grade
- Nuclease free water (non DEPC treated)

Laboratory Equipment and Consumables

- Cepheid SmartCycler® Diagnostic System which includes:
  - I-CORE™ blocks
  - Mini-centrifuge
  - Plastic sample racks
  - Computer (including monitor from Cepheid)
  - Cepheid SmartCycler® Diagnostic Operator Manual
  - Cepheid SmartCycler® Dx Software (comes pre-loaded on computer from Cepheid)
    - Tube Puller

  - Cepheid 25 µL reaction tubes for Cepheid SmartCycler®
  - Barcode reader (optional)
- Printer
- 1.5 or 1.7 mL polypropylene microcentrifuge tubes, certified DNase- and RNase-free (Axygen MCT-175-C-S or equivalent)
- Vacuum System
- Qiagen® VacConnectors
- Qiagen® VacValves
- Vacuum source capable of drawing -800 to -1000 mbar
- Qiagen® Vacuum Manifold
- Qiagen® Vacuum Regulator
- Omni Homogenizer
- Omni Adaptor
- Omni Tip™ Disposable Probes for Omni Homogenizer
- 8 mL and 14 mL polypropylene culture tubes for homogenization.
- Disposable forceps
- Scale capable of weighing milligram amounts
- Weighing paper or boats
- Calibrated pipettors:
  - 1 pipette capable of measuring 5 µL
  - 1 pipette capable of measuring 10 µL
  - 1 pipette capable of measuring 50 µL
  - 1 pipette capable of measuring 400 µL
  - 1 pipette capable of measuring 700 µL
  - 1 additional 1000 uL pipettor capable of measuring 200 µL to 800 µL (used to add ethanol-homogenate mix to column)
- Pipette-Aid
- Disposable serological pipettes (10 mL and 25 mL)
- Mini-centrifuge (optional)
- Rocker
- Aerosol resistant, DNase- and RNase-free tips to fit pipettors
- Microcentrifuge (Eppendorf model 5415D or equivalent)
- Vortex (Vortex Genie 2, Model A 560 or equivalent)
- Scalpel and blades
- Powder-free disposable gloves

WARNINGS AND PRECAUTIONS

1. For *in vitro* diagnostic use.
2. Before testing samples, read the entire contents of these Instructions for Use and obtain Veridex training. Users should successfully pass the Veridex Proficiency Testing consisting of at least 4 BLN assay runs with a minimum passing score of 96%.
3. Qualify all equipment prior to use. Refer to the Cepheid SmartCycler® Diagnostic Operator Manual for installation qualification instructions.

4. As a quality assurance measure during deployment of the GeneSearch™ BLN testing method, laboratories should monitor the rate of same-node discordances observed between test results and permanent section histology results.
5. Do not use expired reagents. Do not use damaged reagent kits.
6. Avoid contaminating lymph nodes with breast tissue. Normal and benign breast tissue can yield false positive assay results. Avoid contaminating lymph nodes with primary tumor tissue as it may produce a false-positive test result.
7. The presence of excess fat surrounding the lymph node tissue decreases the sensitivity of the assay and may result in an 'invalid' test result.
8. Lymph nodes from patients diagnosed with lymphoma may give false positive assay results.
9. Fixing tissue in formalin prior to testing will result in an 'invalid' test result.
10. Do not pool separate lymph nodes as it may result in a loss of assay sensitivity and cause an erroneous result.
11. Processing at ambient temperatures above 30°C may result in a loss of assay sensitivity.
12. Avoid microbial contamination of reagents. Additionally, RNA is susceptible to degradation. It is important to maintain ribonuclease-free conditions.
13. Use caution in placing the SmartCycler® tubes into the instrument. If tubes are not placed into the instrument in the order specified via the software, the run will be invalid.
14. Environmental contamination of the PCR laboratory by PCR products can cause erroneous results. After running the amplification reaction, do not open sample or control tubes under any circumstances in the PCR lab/area to avoid amplicon contamination. If possible, use different laboratory areas to prepare the reaction mix and perform the PCR amplification thus minimizing the possibility of amplicon contamination. Use of a laminar flow hood during assembly of the PCR reactions will minimize the possibility of contamination. After each run, clean the work areas (including applicable equipment) and any spills using fresh 10 % bleach/water solution (let sit for 15 minutes) followed by a water rinse.
15. In cases where other PCR tests are also conducted by the laboratory and open-tube assays are present, use separate and segregated working areas for specimen preparation and amplification/detection activities. Dedicate supplies and equipment to each area and do not move supplies or equipment from one area to another. Always wear gloves and gowns/lab coats and change them before moving from one area to another or before manipulating reagents.
16. All personnel should follow universal precautions and use laboratory safety equipment (i.e. safety glasses, laboratory coat, gloves). If available, a fume hood should be used for preparing sample preparation kit reagents, and a biosafety cabinet should be used for handling biological material. If a hood is not available, consider using a face shield during the homogenization step.
17. Avoid contaminating reagents with bleach. Contamination will cause erroneous results.

18. Contamination of reagents may occur if vial caps are interchanged. Ensure that colored caps are returned to the vial with the same label color. Only one vial should be open at a time.
19. Results of the assay should be used in conjunction with all clinical information derived from diagnostic tests, physical examination, and complete medical history in accordance with appropriate patient management procedures. Use the Run Report, not the Patient Report, to view and report patient results.

- **Warning**! All biological specimens and other materials coming into contact with the specimen(s) are considered biohazardous. Handle as if capable of transmitting infection. Treat and dispose of waste using proper precautions and in accordance with local, state, and federal regulations. Never pipette by mouth.
- **Warning**! Homogenization Buffer contains guanidine thiocyanate: Risk and safety phrases * R20/21/22 (Harmful by inhalation, in contact with skin and if swallowed), R32 (Contact with acids liberates very toxic gas), S13 (Keep away from food and drink), S26 (In case of contact with eyes rinse immediately with plenty of water and seek medical advice), S36 (Wear suitable protective clothing), S46 (If swallowed seek medical advice immediately and show label or container). These materials are harmful. Wear appropriate protective clothing when working with these materials. Guanidine thiocyanate can form highly reactive compounds when combined with bleach. Use a suitable laboratory detergent and water to clean spills followed by bleach solution if decontamination is required.
- **Warning**! Wash Buffer 1 contains 10 % ethanol and is considered flammable (R10, Flammable). Do not use around open flames.
- **Warning**! Some of the reagents contain Proclin 300 preservative. Symptoms of overexposure to Proclin 300 may include irritation of skin, eyes, mucous membranes and upper respiratory tract.
- **Warning**! Wash Buffer 2 contains sodium azide. Azide compounds should be flushed with large volumes of water during disposal to avoid deposits in lead or copper plumbing where explosive conditions can develop. Harmful if swallowed. After contact with skin, wash with plenty of soap.

REAGENT STORAGE AND HANDLING

GeneSearch™ Breast Lymph Node (BLN) Test Kit Storage and Handling

Store at –15°C to –25°C, before opening and after opening. Kit is stable for 2 hours at room temperature.

Follow the instructions for mixing of reagents provided in the "RT-PCR" section of this document. Thaw and mix reagents prior to use. The GeneSearch™ Breast Lymph Node (BLN) Test Kit should be removed from the freezer at least 20 minutes prior to use to allow for complete thawing. Utilize a rocker for thawing and mixing reagents, if available. If a rocker is not available, mix thawed reagents by inverting the vials (cap down) and pulse-vortexing each reagent three times. Spin each reagent briefly in the microcentrifuge or mini-centrifuge to remove any liquid from caps before opening. Inadequate mixing of the PCR reagents prior to use can affect the sensitivity and reproducibility of the assay and cause

erroneous assay results.
Acceptable functional performance for the GeneSearch™ BLN Test Kit was observed following up to 12 freeze-thaw cycles.
Protect reagents from exposure to direct light.
When properly stored, reagents are stable until the expiration date printed on the kit label. Do not use expired reagents.
The GeneSearch™ BLN Test Kit components are manufactured and tested as a master lot. Do not interchange reagents from different lots.

- After completing 30 reactions, any residual reagents must be discarded.
- Once opened, the GeneSearch™ BLN Test Kit, if stored correctly, has been shown to be stable for 6 months, or until kit expiration date, whichever comes first.
- The volume of Master Mix, Enzyme Mix, and Sample or control added to the PCR reaction affects the Ct value obtained. Care should be taken to ensure that 10 µL of Master Mix and Enzyme Mix are added to the reaction and 5 µL of sample and/or control are added. The assay can tolerate variations in the volume of any one reactant up to 25%, however, variations in the volumes of multiple reactants (such as those that would result from poor pipetting technique) will cause variations in Ct values that may negatively impact assay results.
- Once PCR reactions are assembled (Master Mix, Enzyme Mix, and sample are added to the Cepheid tube) the thermal cycling should be initiated within 30 minutes. Holding the assembled reactions for longer than 30 minutes before initiation of thermal cycling will result in a loss of assay sensitivity.

GeneSearch™ RNA Sample Preparation Kit Storage and Handling

- Store at 15°C to 25°C, i.e. ambient temperature.
- When properly stored, reagents are stable until the expiration date printed on the kit label. Do not use expired reagents.
- Once opened, the GeneSearch™ RNA Sample Preparation Kits can be stored at 15 °C to 25 °C (the recommended storage temperature) for a period of 30 days or until kit expiration date, whichever comes first.
- Working reagents prepared for the Sample Preparation Kit (working Homogenization Buffer and working Wash Buffer 2) can be stored for a period of 30 days or until kit expiration date, whichever comes first.
- The RNA Sample Preparation Kit components are manufactured and tested as a master lot. Do not mix and match reagents from different lots.
- After completing 10 purifications, any residual reagents must be discarded.
- Eluted RNA is stable at ambient conditions (15 °C to 25 °C) for up to 60 minutes.
- Holding homogenates (homogenized tissue solution) for more than 60 minutes at room temperature may result in a loss of assay sensitivity for some samples. Homogenate solutions should not be held at room temperature for more than 60 minutes. If homogenates will not be tested immediately, solutions should be frozen at –65°C or below until needed.

- The ratio of homogenate to 70% ethanol should be 1:1 (equal parts of homogenate and 70% ethanol solution). Be careful to accurately measure both solutions to ensure that the ratio remains 1:1. Ratios of 1.33, 1.25, 1.0, 0.8 and 0.75 homogenate-to-ethanol were evaluated for the affect on assay performance. A ratio of 0.75 or less (300 µL homogenate:400 µL ethanol) was found to give unacceptable results.
- The 70 % ethanol solution must be remixed prior to use. Failure to mix before use may decrease the sensitivity of the assay.

TISSUE AND ASSAY INTERMEDIATES STORAGE AND HANDLING

- If lymph node tissue will not be tested within 45 minutes after removal from the patient, the tissue should be snap-frozen in liquid nitrogen and stored frozen at –65°C or below until testing occurs. If the sample is frozen, it should not be allowed to thaw prior to homogenization. Keep samples frozen on dry ice during weighing and sectioning until homogenization. Once Homogenization Buffer is added, homogenize immediately.
- Lymph node tissue that is homogenized immediately upon removal from the patient can be stored frozen at –65°C or below for testing within 21 days. Frozen homogenates should be thawed completely at room temperature (until all ice crystals disappear) and mixed prior to use.
- It is not recommended that homogenate-ethanol mixes be stored for use at a later date. These intermediates are stable for up to four hours at ambient temperatures. If they will not be used within 4 hours of being created from the homogenates, they should be discarded and new homogenate-ethanol mixes should be created at the time of RNA purification.
- RNA can be purified from fresh tissue immediately upon removal from the patient and the RNA can be stored frozen for up to nine weeks for testing at a later date. If frozen RNA samples will be used they should be thawed and mixed prior to use.
- If tissue or assay intermediates (homogenates, RNA) will be shipped, they should remain frozen and be shipped on dry ice.

TEST PROCEDURE

Preparation of Working Reagents

Homogenization Buffer

To prepare Working Homogenization Buffer, add 1.0 mL β-mercaptoethanol (β-ME) to one bottle of Homogenization Buffer and mix well. Reagent is stable for 30 days after β-ME addition at ambient temperature. Record expiration date on container. Homogenization Buffer may form a precipitate upon storage. If necessary, redissolve by warming to 30 to 37°C and mix gently by inversion until the precipitate dissolves. Return to ambient temperature before use.

Warning! β-ME is toxic; wear appropriate protective clothing and dispense in a fume hood, if available.

Wash Buffer 2

To prepare Working Wash Buffer 2, add 8 mL absolute 200-proof ethanol to Wash Buffer 2 concentrate in its container and mix well. Reagent is stable when stored at ambient temperature for 30 days after ethanol addition. Record expiration date on container.

70% Ethanol

To prepare 70% ethanol, add 7 mL 200-proof molecular biology grade ethanol to 3 mL of nuclease-free water. Ensure the solution is mixed well prior to use. Incomplete mixing of the 70% ethanol solution prior to use may cause erroneous assay results.

Specimen Collection

1. Care must be taken to minimize the contamination of the lymph node with breast or primary tumor tissue, as this may cause a false positive result in the assay. Clean surgical instruments and surgical trays must be used.
2. Lymph node tissue should be placed in a fresh transport container after excision, labeled appropriately, and immediately transported to the pathology cut-in area.
3. The lymph node should be prepared as soon as possible to minimize RNA degradation. If tissue must be held for any period of time before processing (e.g., while waiting for additional lymph nodes), keep the tissue on the weighing paper until processing begins. DO NOT place the tissue in the homogenization buffer and allow it to remain for any period of time. Each lymph node should be processed as a separate specimen. Do not use any tissue fixatives on the lymph node prior to preparation. Tissue is stable for 45 minutes at room temperature after removal from the patient. If tissue will not be homogenized within 45 minutes it should be flash frozen in liquid nitrogen and placed in the freezer at -65°C or below until testing will commence. If frozen tissue will be used, do not allow tissue to thaw before homogenization. Tissue should be placed on dry ice during weighing and sectioning.
4. Before preparing the lymph node, clean the cutting board and spread a fresh disposable surface on the cutting board. Put a fresh blade on the scalpel and use a fresh pair of gloves. Change gloves, scalpel blades, forceps, and cutting surface between lymph nodes.

   Note: This is essential to minimize sample cross-contamination.
5. Remove the lymph node from its container with gloved hands and clean forceps, and place onto the fresh disposable surface.
6. Clean the lymph node of any fibroadipose tissue (fat) following standard procedures for the laboratory. Fat is a known interfering substance in the assay. Check for and remove any non-lymph node material.
7. Remove tissue sections that are required for routine histopathology testing.

Note[1]: During clinical trials 50% of the lymph node was tested with the GeneSearch™ BLN Assay and the remaining tissue was submitted for histology, a portion of which was sampled and tested to obtain the performance results as detailed in the "Clinical Performance" section below. The lymph node should be cut using the node cutting technique utilized in the clinical

study (see below for node cutting technique) in order to obtain the performance described in the "Clinical Performance" section.

Note[2]: Lymph nodes from patients diagnosed with lymphoma may cause a false positive result.

Sample Preparation

Homogenization

Homogenization Buffer and Wash Buffer 2 must be prepared as described in the "Preparation of Working Reagents" section before proceeding further.

1. Determine the weight in milligrams (mg) of each lymph node to be tested in the GeneSearch™ BLN Assay. Place a fresh piece of weigh paper on the balance, tare, and weigh the lymph node.
2. If a lymph node is greater than 2 to 3 mm in any dimension, use a fresh scalpel blade to mince it into pieces approximately 2 to 3 mm in diameter. Care should be taken to avoid contamination of the tissue during processing.

   Do not place tissue into homogenization buffer until immediately before homogenization. Once tissue has been introduced to the homogenization buffer, homogenize without delay.
3. Label tubes for homogenization in such a way that source specimens can be identified (with Patient ID and Node ID, if applicable).
4. Add Homogenization Buffer to the homogenization tube (8 mL or 14 mL polypropylene culture tube; for Homogenization Buffer volume below 4 mL use an 8 mL tube, otherwise use a 14 mL tube). Use Table 1 to determine the required volume.

Table 1. Volume of Homogenization Buffer Required

Tissue Weight (mg)	Homogenization Buffer (mL)	Tube Size (mL)
3 – 149	2	8
150 – 199	3	8
200 – 249	4	8
250 – 299	5	14
300 – 349	6	14
350 – 399	7	14
400 – 449	8	14
450 – 499	9	14
500 – 550	10	14
> 550	See note below	

Note: Lymph nodes weighing greater than 550 mg will not be adequately homogenized using the recommended system. They should be divided into equivalent parts prior to homogenization and each part should be homogenized, purified, and assayed as an individual specimen.

5. Using clean forceps, transfer the tissue into the Homogenization Buffer.

6. Place a new homogenization probe into the Omni homogenizer.
7. Homogenize each lymph node completely (typically 30 to 60 seconds) using the highest homogenizer setting available. Use a new homogenizer probe for each lymph node.
8. Process the homogenate as described in the "RNA Purification" section.

   Note: Once the homogenates are created, they are stable at ambient temperature for up to 60 minutes. If they will not be tested immediately, they should be frozen at –65°C or below until RNA purification will begin. If previously frozen homogenates will be used in the assay, ensure that the homogenates are completely thawed at room temperature (no ice crystals visible) and mixed prior to use.
9. Dispose of the homogenization probes.

RNA Purification

Multiple homogenates can be processed using this procedure. Always ensure that the vacuum is turned to the OFF position prior to the application of a solution to RNA Spin Columns during RNA purification.

1. Label tubes in such a way that source specimens can be identified (with Patient ID and Node ID, if applicable).
2. Add 400 µL of homogenate to 400 µL of 70% ethanol in a 1.5 mL tube and mix by vortexing for 10 seconds.

   Note[1]: The ratio of homogenate to 70% ethanol should be 1:1 (equal parts of homogenate and 70% ethanol solution). Be careful to accurately measure both solutions to ensure that the ratio remains 1:1. The 70% ethanol solution must be remixed prior to use. Failure to mix before use may decrease the sensitivity of the assay.

   Note[2]: The homogenate-ethanol mix solutions can be held for up to 4 hours at ambient temperature. If RNA purification will not be initiated within 4 hours of creation of the homogenate-ethanol mix the homogenate-ethanol mix should be discarded and a new mix should be created from the original homogenate solution.
3. For each sample, attach a VacValve onto a Vacuum Manifold and a disposable VacConnector to each valve.
4. Attach a RNA Spin Column on to the VacConnector, leaving the cap open. Ensure that each VacValve is closed.
5. Label each column with Patient ID and Node ID, if applicable. Aliquot each homogenate/ethanol mix from step 2 onto a separate RNA Spin Column (Column). The volume of homogenate/ethanol mix to be added to the column is based on the original tissue amount and is provided in Table 2.

Table 2. Volume of Homogenate/Ethanol Mix Required

Tissue Weight (mg)	Volume of Homogenate-Ethanol Mix (µL)
3 – 39	700
40 – 49	500
50 – 59	400
60 – 69	350
70 – 79	300
80 – 89	250
90 – 99	225
≥ 100	200

6. Turn VacValves to the ON position and apply vacuum (-800 to -1000 mbars) until sample is filtered (approximately 30 seconds). The vacuum must remain between -800 and -1000 mbars throughout the filtration.
7. Turn VacValves to OFF position. Add 700 µL of Wash Buffer 1 to each column. Turn VacValves to ON position and allow the solution to filter through the column. Turn VacValves to OFF position.
8. Add 700 µL of Wash Buffer 2 to each column. Turn VacValves to ON position and allow the solution to filter through the column. Turn VacValves to OFF position.
9. Remove each column from the Vacuum Manifold and place into a 2 mL collection tube (supplied with the column).
10. Centrifuge the tube(s) containing the column(s) for 30 seconds at > 10,000 RPM in a microcentrifuge.
11. Discard the collection tube(s). Put each column into a new collection tube (1.5 to 1.7 mL polypropylene microcentrifuge tube).
12. Add 50 µL of RNase-free water directly to the filter membrane in the center of each column.
13. Centrifuge at > 10,000 RPM for 30 seconds in a microcentrifuge.

    Before continuing to step 14, ensure that the collection tube has been labeled in such a way that the source specimen can be identified (e.g., Patient ID and Node ID).
14. Discard the column(s). Approximately 50 µL of eluted RNA solution will be contained in each collection tube.
15. Proceed immediately to RT-PCR.

    Note: The eluted RNA solution is stable for up to 60 minutes at ambient temperature. If the RNA solution(s) will not be tested immediately, they should be frozen at –65°C or below until the assay can begin. If previously frozen RNA solutions will be used in the assay, ensure that the RNA solutions are completely thawed (no ice crystals visible) and mixed prior to use.

RT-PCR

It is critical that tissue or other environmental materials do not contaminate the assay reagents and components coming into contact with reagents and specimen. Ensure the work area is clean and free of materials not required to process the GeneSearch™ BLN Assay. Change gloves prior to assembling the PCR tests. Use only PCR-quality disposables and discard if there is a possibility of contamination. A dedicated pipette for the PCR area is recommended.

1. Thaw and mix the reagents before processing.

   Note: Inadequate mixing of the PCR reagents prior to use can affect the sensitivity and reproducibility of the assay.

   Centrifuge the RNA solution tubes at high speed briefly to collect contents at the bottom of the tube. The RNA solution tubes can be left at room temperature for up to 2 hours.

2. Turn on the computer and SmartCycler® instrument. Log in, input Patient ID, Node ID, and GeneSearch™ BLN Test Kit lot number.

3. Obtain the required number of Cepheid PCR reaction tubes (one for the Negative Control, one for the Positive Control and one for each patient sample). Label each reaction tube to clearly identify the source specimen and place it in the rack. A maximum of six (6) samples can be tested in each run.

   Note: Accurate pipetting is essential for optimal performance of the BLN Assay.

4. Using a fresh pipette tip for each tube, add 10 µL Master Mix to each reaction tube.

5. Using a fresh pipette tip for each tube, add 10 µL Enzyme Mix to each reaction tube.

6. Using a fresh pipette tip, add 5 µL Negative Control (Purple Cap) to the bottom of the reaction tube labeled "Negative Control." Close the tube, ensuring the cap has completely snapped in place.

7. Using a fresh pipette tip, add 5 µL Positive Control (Green Cap) to the bottom of the reaction tube labeled "Positive Control." Close the tube, ensuring the cap has completely snapped in place.

8. Using a fresh pipette tip for each specimen, add 5 µL of specimen to the bottom of their respective reaction tubes. Close the reaction tubes, ensuring the caps have completely snapped in place. Proceed immediately to the next step.

9. Centrifuge the reaction tubes for 15 to 30 seconds in the SmartCycler® mini-centrifuge.

10. Set up a run in the SmartCycler® software following the instructions provided in the Cepheid SmartCycler® Diagnostic Operator Manual. Assay run parameters are a part of the protocol selected when the run is set up and are provided on the protocol disk provided by Veridex.

11. Place the reaction tubes in the SmartCycler® instrument in the locations selected when the run was set up. When handling SmartCycler® reaction tubes, hold by the ribbed upper portion of the tube. Avoid touching the optical detection windows at the bottom of the tubes to ensure an accurate optical reading. Confirm that the appropriate tubes are placed

in the appropriate sites of the instrument by checking the site map. Ensure that tubes are firmly seated in SmartCycler® unit.

12. Close the lids on the SmartCycler® instrument and initiate the run. Assay run parameters will be provided with the SmartCycler® software.
13. Ensure that assay setup is done rapidly. Sensitivity of the assay may be reduced if the cycling protocol is not initiated within 30 minutes of assembling the reaction.
14. Following completion of the run, remove the Cepheid PCR reaction tubes using the Tube Puller provided. Discard the tubes **without opening** the tubes.

Note! In order to avoid environmental contamination by PCR amplicons, it is critical that the reaction tubes NOT be opened after beginning the RT-PCR reaction. If a tube inadvertently opens, immediately decontaminate by wiping the area with a fresh 10% bleach solution and rinsing with water 15 minutes later. After decontamination, set up reactions with Positive and Negative controls to demonstrate a lack of residual environmental contamination.

Retesting

Testing may be repeated based on the mode of failure.

External Control Failures: If a run is invalidated due to failure of external controls, the user can repeat the assay using the residual RNA sample from patient lymph node(s) and external controls from the GeneSearch™ BLN Test Kit.

Internal Control Failures: If a sample fails because all markers including the internal control are negative for a given patient (from one or more lymph nodes), RNA can be re-purified from the respective homogenate(s) and the assay can be repeated.

When repeat testing is necessary, the later run should be started as soon as the results are available from the first run. The tissue homogenate and purified RNA are stable up to 60 minutes at ambient temperature when proper caution is exercised during their preparation.

QUALITY CONTROL

External Controls

External controls are provided for MG and CK19 (Positive Control) and for PBGD (Negative Control). These controls must be included with each run. External controls consist of linearized plasmids containing sequences capable of being amplified and detected by the primers and probes used in this GeneSearch™ product. The controls monitor reagent quality and instrument performance as they relate to assay performance.

Internal Control

The internal control consists of detection of mRNA from a constitutively expressed gene (PBGD) in lymph node tissue as a control against false negative results. The results of this control are obtained as one of the multiplexed gene markers in the specimen reaction tube. This control monitors the sample quality, sample preparation and assembly of the RT-PCR reaction in the specimen reaction. It is possible that extremely high expression of the cancer markers will inhibit detection of the internal control. As a result, assays in which one or both

cancer markers are positive in at least one lymph node are considered valid, regardless of the result observed for the internal control.

Contamination Controls

The Positive Control and Negative Control also serve as contamination controls. The Positive Control does not contain the target sequence for the internal control and serves as its contamination control. The Negative Control does not contain MG or CK19 target sequences and serves as their contamination controls. The contamination control system is designed to detect environmental contamination or nonspecific products that could result in incorrect assay results.

Interpretation of Results

The software will generate results in the form of a report. The results presented on the "Run Report" (not the "Patient Report") should be used for reporting patient results. The Run Report provides information in the sections and corresponding field names as shown in Table 3.

Table 3. Report Sections and Field Names.

Refer to the Cepheid SmartCycler® Dx Operators Manual.

Section	Field Names
Run Information	Run Name, User Name, Run Status, Start and Finish Times, Software Version
Assay Information	Assay Name and Version, Kit Lot Number and Expiration date, Assay Status, Assay Type
Patient Results	Number of Instrument Sites used per Patient (referring to the number of samples per patient), Patient ID, Patient Result, Number Positive
Sample Results*	Site ID, Patient ID, Sample ID**, Assay Result for each site, IC (Internal Control) result by site, any Warning or Error Code, Notes inputted by user, Marker 1 and Marker 2 results
Error(s)	Error(s) or Warnings
Instrument(s)	Instrument and Serial Number
Growth Curve	Plot of fluorescence versus cycle number. The increase in fluorescence is proportional to the amount of amplicon generated and is used to determine the cycle threshold.

* Sample Results = Node Results

** Sample ID = Node ID

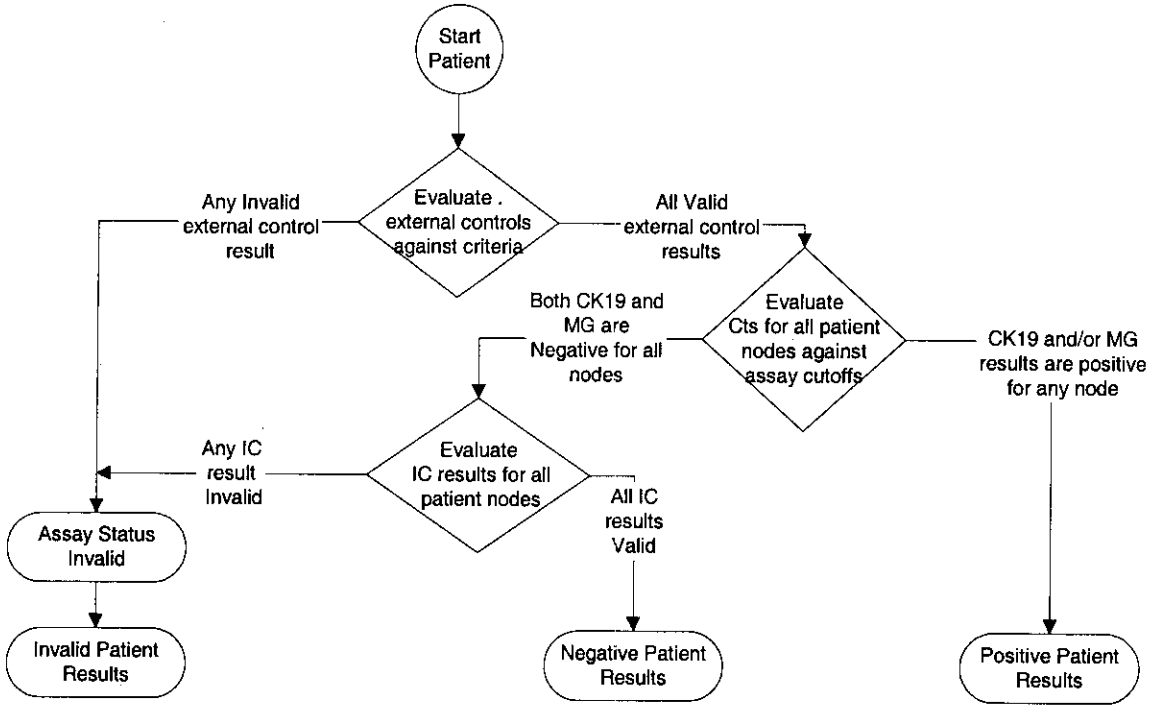
Each patient result in a run is displayed in the Run Report as determined by utilizing the decision tree depicted in Figure 1 below. The expected qualitative Patient Result will be "Positive", "Negative", or "Invalid" based on the Ct values obtained for each marker in each sample.

Cutoff values

Cutoff values have been specified for all markers. In order to assess patient results, external control values must be valid. Samples with Ct values less than or equal to one or both of the cutoff values for MG or CK19 are considered positive. If the MG and CK19 Ct values are above their cutoffs the Internal Control Ct value must be below its cutoff for MG and CK19 to be considered negative. If the MG and CK19 Ct values are negative and the Ct value for the Internal Control gene is greater than or equal to its cutoff, then the result for that sample will be considered "invalid".

The Cutoff Ct values are as follows: MG ≤ 31, CK19 ≤ 30, Internal Control < 36.

Figure 1. Decision tree to determine patient result.



If the Assay Status field is "Valid", then the Patient Result field in the Patient Results section shows the test result, be it "Positive" or "Negative." This result is per patient and not per lymph node. If at least one of the lymph nodes being tested for a patient is positive for either assay marker, then the result reported in the Patient Result field is "Positive", regardless of the results obtained with other samples from that patient. However, if the Patient Result is not "Positive" and the IC result from one or more of the patient's lymph nodes is "Invalid", then the Patient Result is "Invalid" (this applies also if the patient's node are tested in more than one run). The Patient Result field in the Patient Results table provides an objective readout of patient status requiring no subjective interpretation by the user.

Note[1]: Ensure the validity of the Positive and Negative Controls before reporting patient results. Results from runs with one or more Invalid controls must not be reported.

Note[2]: Use the Run Report, not the Patient Report.

If the Assay Status field in Assay Information is "Invalid", then the Patient Result is "Invalid." In such cases, assay results obtained in that run are invalid and must not be reported. Invalid assay run or instrument error codes or warnings are flagged on-screen and on reports. Before reporting GeneSearch™ BLN Assay results, always verify that the "Assay Status" is "Valid". Refer to the Cepheid SmartCycler® Diagnostic Operator Manual for printing results.

LIMITATIONS

- The lymph node should be cut using the node cutting technique utilized in the clinical study (see below for node cutting technique) in order to obtain the performance described in the "Clinical Performance" section.
- The performance of the GeneSearch™ BLN Assay was verified using the procedures provided in these "Instructions For Use" only. Modifications to these procedures may alter the performance of the assay.
- The performance of the GeneSearch™ BLN Assay for patients receiving neoadjuvant treatment has not been established.
- Contamination of the specimen with breast tissue (normal, benign, or malignant) or breast lymph node tissue from another patient may cause erroneous results. Care must be taken in all phases of sample processing to avoid contamination.
- The presence of fat on/in the lymph node tissue decreases the sensitivity of the assay and may result in an "invalid" test result.
- Lymph nodes from patients diagnosed with lymphoma may give false-positive assay results.
- Avoid contaminating lymph nodes with primary tumor tissue as it may produce a false-positive test result.
- Tissue less than 50 mg in weight may yield a higher Invalid test rate with the GeneSearch™ BLN Assay.
- The GeneSearch™ BLN Assay has been designed to work with SLND procedures yielding no more than 6 samples per run. 2 runs may be performed simultaneously or sequentially accommodating a total of 12 samples per Cepheid.
- The GeneSearch™ BLN Assay is not designed for use with formalin-fixed tissue.
- GeneSearch™ BLN Assay results should be used in conjunction with all clinical information derived from diagnostic tests (i.e. imaging or laboratory tests), physical examination, and full medical history in accordance with appropriate patient management procedures.

The GeneSearch™ BLN Assay has been designed, optimized, and tested for use with the GeneSearch™ RNA Sample Preparation Kit and the Cepheid SmartCycler® System.

INTERFERING SUBSTANCES

Potentially interfering substances include, but are not limited to the following:

- Fat, if present, should be trimmed from tissue sections being used in the assay to avoid erroneous results. A mixture of fat and positive lymph node tissue were processed at different ratios (Fat:Positive Lymph Node 100:0, 75:25, 50:50, 25:75 and 0:100). Fat was found to have an effect on the Assay Result when present in a higher amount than the positive lymph node sample in the mixture (75 fat: 25 lymph node).
- Patients with a previous reported history of lymphoma may not be good candidates for the assay. Lymph nodes without histological evidence of metastatic breast cancer were obtained from patients diagnosed with lymphoma and tested by Veridex using the GeneSearch™ BLN Assay. Of eight lymph nodes tested, one false positive result was observed.

  In clinical studies of this assay (n = 720), 65 patients reported a previous history of another cancer type. Of the 16 patients of this group found positive by histology, the assay was positive in 15, (sensitivity = 93.8%). Of the 49 patients found to be negative by histology, the assay was negative in 47 (specificity = 95.9%) and positive in 2 (false positive, 2/49 or 4.1%). One of 4 patients reporting a history of lymphoma and 1 of 14 reporting a history of melanoma accounted for the 2 false positives. While the overall clinical performance of the assay in patients reporting previous history of cancer is comparable to the performance in those reporting no history of cancer, it is possible that previous or concurrent diagnosis of other cancers, particularly lymphoma, could result in a false positive assay result.
- Contamination with breast tissue may cause a false positive result. Tissue homogenate containing 50 mg/mL, 1 mg/mL, 0.02 mg/mL, 0.4 µg/mL, and 0.008 µg/mL of breast tissue was tested. Positive results (3 of 5) were obtained only for the tissue concentration of 50 mg/mL.
- Contamination with primary breast tumor tissue may cause a false-positive result. Five of five tissue specimens of breast tumor tested positive. In a separate study, tissue homogenate containing 50 mg/mL, 1 mg/mL, 0.02 mg/mL, 0.4 µg/mL, and 0.008 µg/mL of tumor tissue was tested. Positive results were obtained only at the two highest concentrations: at 50 mg/mL, 4 of 5 samples were positive. At 1 mg/mL, 2 of 5 samples were positive.
- GeneSearch™ BLN Assay performance was analyzed in the presence of blood, tissue marking dyes, tracing dyes, and technitium^{99} (Tc^{99}). Assay performance was not affected by the presence of any of these materials.

ANALYTICAL PERFORMANCE

Precision

Precision is a measurement of the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple samples of a homogeneous sample. The degree of scatter in multiple measurements of a homogeneous sample is due to several factors such as the operator performing the measurement, the lot number of the material with which the sample is measured, the day on which the measurement is taken, and the variability inherent

in the measurement system itself. It does not assess biological variability within the specimen or pre-analytical variability in sample preparation.

The precision of the GeneSearch™ BLN Test Kit was determined using a protocol similar to that recommended in the Clinical Laboratory Standards Institute Evaluation of Precision Performance of Quantitative Measurement Methods; Approved Guideline-Second Edition (EP05-A2). Two samples and two assay controls were tested on the GeneSearch™ BLN Test Kit using three operators testing both samples and controls on three lots of GeneSearch™ BLN Test Kits each day for eight days with two runs per day and with two replicates of each sample and control tested on each run.

The Ct values obtained for each applicable marker in each sample were analyzed to determine the standard deviation of the measurements. The standard deviation is divided by the mean Ct value for each marker in each sample and multiplied by 100 to determine the percent Coefficient of Variance (%CV), or degree of scatter in the data.

$$\%CV = (\text{Standard Deviation/Mean}) \times 100$$

The Within Run Precision expresses the degree of scatter in measurements taken on a given sample within the same run. The Total Precision expresses the degree of scatter in the measurements on a given sample across all operators, all days, all lots, and all runs.

Total assay precision was estimated using the model provided in EP05-A2. The formula used to estimate total precision was

$$S_T = \sqrt{S_{dd}^2 + S_{rr}^2 + S_{wr}^2}$$

where S_T = total standard deviation, dd = between day, rr = between run and wr = within run. The results are provided in Table 4.

Table 4. Total Run Precision Excluding Lot and Operator

Total Precision (%CV) Excluding Lot and Operator (S_T/Mean Ct)				
	Positive Sample	Negative Sample	Negative Control (NC)	Positive Control (PC)
PBGD	2.9%	4.3%	1.9% (3.1%) [c]	NA [b]
MG	3.1%	NA [a]	NA [b]	1.6 % (6.0%) [c]
CK19	1.5%	NA [a]	NA [b]	1.6% (5.6%) [c]

a. MG and CK19 were not analyzed, as these markers are not expressed at appreciable levels in a negative sample.
Negative results were obtained as expected in samples not containing these markers.

b. MG and CK19 are not present in the Negative Control. PBGD is not present in the Positive Control.
Negative results were obtained as expected in samples not containing these markers.

c. Values, if results from samples where control was not added (user error) to the reaction, are included in the analysis.

Lot-to-lot and operator-to-operator variability were also considered in the reproducibility study design. The formula used to estimate total precision in this case was

$$S_T = \sqrt{S_{dd}^2 + S_{rr}^2 + S_{wr}^2 + S_{op}^2 + S_{lot}^2}\,.$$

The assay results are reproducible; the values for each sample tested are provided in Table 5.

Table 5. Total Run Precision Including Lot and Operator

Total Precision (%CV) Including Lot and Operator (S_T/Mean Ct)				
	Positive Sample	**Negative Sample**	**Negative Control (NC)**	**Positive Control (PC)**
PBGD	5.6%	5.2%	2.5% (3.4%) [c]	NA [b]
MG	5.5%	NA [a]	NA [b]	1.8% (6.1%) [c]
CK19	2.5%	NA [a]	NA [b]	1.9% (5.6%) [c]

a. MG and CK19 were not analyzed, as these markers are not expressed at appreciable levels in a negative sample. Negative results were obtained as expected in samples not containing these markers.

b. MG and CK19 are not present in the Negative Control. PBGD is not present in the Positive Control. Negative results were obtained as expected in samples not containing these markers.

c. Values, if results from samples where control was not added (user error), to the reaction are included in the analysis.

Linearity

The linearity of an analytical or biological test method is its ability (with a given range) to obtain results that are directly proportional to the concentration of analyte in the sample. The linearity of the GeneSearch™ BLN Test Kit was assessed by preparing samples containing known amounts of *in vitro* transcript (IVT) RNA for each marker, testing these samples on the GeneSearch™ BLN Test Kit, and directly comparing the Ct values obtained for each marker in each sample to the concentration of each marker in each sample by regression analysis.

Linearity is expressed by the equation of the line (Y=mx+b) which results from plotting the Ct value of each marker (Y) versus the concentration of target ('x' expressed as the

$\log_{10}$(copies/μL)) in each sample (regression analysis). The regression line expresses the best prediction of the Ct value based on the concentration.

Samples were prepared by adding IVT RNA in log-fold increments (increases of 10 copies of IVT RNA/μL buffer) and tested on the GeneSearch™ BLN Test Kit. Samples were prepared with IVT RNA for one marker at a time (individual IVT RNA) and in combination with each other (IVT RNA Mix). Regression analysis was performed for each marker for all levels of target that gave detectable signal in the assay. The resultant equations of the lines for each marker and R^2 values are presented in the table below. The assay met the criteria for a linear response between the lowest level detected (shown in Table 6 below) and 10^8 (8 log) copies per μL for all markers tested.

Table 6. Linearity and Detection Limits

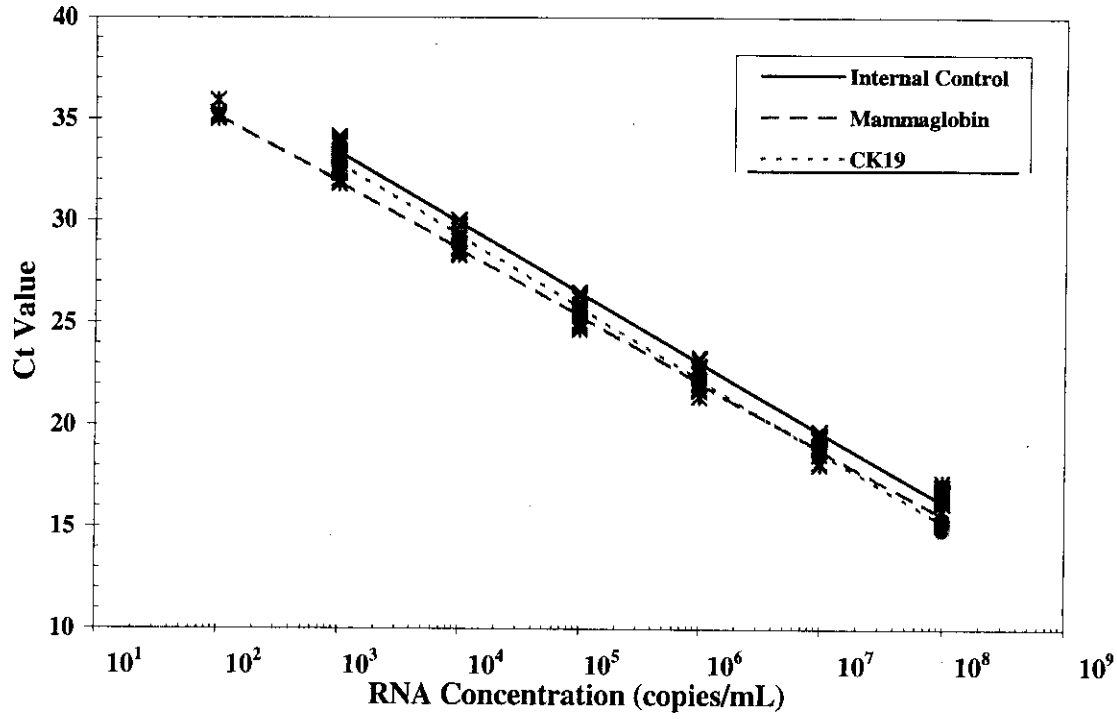
Individual IVT RNA

	Equation of the Line Ct = b+(m * ($\log_{10}$(copies/μL))	R^2 value	Lowest Target Level Detected
IC Ct	PBGD Ct = 44.50 - 3.571 * $\log_{10}$(copies/μL)	0.997	10^3
MG Ct	MG Ct = 41.62 - 3.265 * $\log_{10}$(copies/μL)	0.993	10^2
CK19 Ct	CK19 Ct = 43.62 – 3.559 * $\log_{10}$(copies/μL)	0.999	10^3

IVT RNA Mix

	Equation of the Line Ct = b+(m * ($\log_{10}$(copies/μL))	R^2 value	Lowest Target Level Detected
IC Ct	PBGD Ct = 43.58 - 3.419 * $\log_{10}$(copies/μL)	0.995	10^3
MG Ct	MG Ct = 41.7 - 3.269 * $\log_{10}$(copies/μL)	0.994	10^2
CK19 Ct	CK19 Ct = 43.38 – 3.521 * $\log_{10}$(copies/μL)	0.999	10^3

Figure 2: Linearity and Detection Limits



Limits of Detection

A theoretical limit of detection was calculated using the equation generated by regression analysis during linearity testing. The limit of detection is defined as the number of copies of the target sequence detected at 35.9 Ct, the highest value that can be obtained with the GeneSearch™ BLN Assay thermal cycling protocol. Data is shown in Table 7 below.

Table 7. Detection Limits

Individual IVT RNA

	Equation of the Line $Y = b-(m * (\log_{10}(\text{copies}/\mu L))$	Analytical Detection Limit
IC Ct	PBGD Ct = 44.50 - 3.571 * $\log_{10}$(copies/µL)	$10^{2.4}$
MG Ct	MG Ct = 41.62 - 3.265 * $\log_{10}$(copies/µL)	$10^{1.8}$
CK19 Ct	CK19 Ct = 43.62 – 3.559 * $\log_{10}$(copies/µL)	$10^{2.2}$

IVT RNA Mix

	Equation of the Line $Y = b-(m * (\log_{10}(\text{copies}/\mu L))$	Analytical Detection Limit
IC Ct	PBGD Ct = 43.58 - 3.419 * $\log_{10}$(copies/µL)	$10^{2.3}$
MG Ct	MG Ct = 41.7 - 3.269 * $\log_{10}$(copies/µL)	$10^{1.8}$
CK19 Ct	CK19 Ct = 43.38 – 3.521 * $\log_{10}$(copies/µL)	$10^{2.1}$

GeneSearch ™BLN Assay Reproducibility

Two operators from each of three sites participated in a Reproducibility Study. All operators tested a Veridex-provided reproducibility panel composed of human axillary lymph node tissue homogenate supplemented, when needed, with *in vitro* transcript of high or low levels of MG and/or CK19. There were a total of four panel members, one being negative for MG and CK19. Starting from the RNA isolation step, each operator tested panel samples in duplicate in each run using three different lots of the GeneSearch™ BLN Test Kit. Samples were tested with the same lot of reagents on two separate days by each operator. The study design resulted in a total of 72 planned replicate results for each of the four panel members across all lots, sites, days, and operators. The GeneSearch™ BLN Assay results were in 100% agreement with the known presence or absence of target for all individual markers (PBGD, MG and CK19). Percent Coefficient of Variations (CVs) for all marker Ct values were ≤ 6.82 % for intra-run, inter-run, inter-site, inter-operator and inter-lot analyses. Standard deviations (SDs) were ≤ 1.88 in all cases.

These data show that GeneSearch™ BLN Assay results are reproducible across sites, operators, lots, days and within runs.

Table 8. Variability of Ct Values by Variability Sources. Ct values of invalid MG and CK19 results due to IC failure are not included in the analysis.

Panel		Agreement		Intra-Run		Inter-Run		Inter-Site		Inter-Oper		Inter-Lot	
		%	Mean	SD	%CV	SD	%CV	SD	%CV	SD	%CV	SD	%CV
A (n=74)	MG: Neg	100	39.92	0.45	1.12	0 [a]	0	0 [a]	0	0.14	0.36	0	0
	CK19: Neg	100	37.44	1.88	5.03	1.60	4.28	1.11	2.96	0 [a]	0	0.87	2.31
	PBGD: Pos	100	28.47	0.90	3.15	0.41	1.45	0 [a]	0	0.39	1.37	0.94	3.31
B (n=72)	MG: Low Pos	100	23.19	0.51	2.18	0.17	0.73	0	0	0.29	1.26	0.71	3.05
	CK19: Neg	100	39.54	1.02	2.57	1.06	2.69	0.48	1.22	0.72	1.82	0.18	0.44
C (n=74)	MG: Neg	100	37.98	1.11	2.93	0.30	0.79	0.31	0.82	0 [a]	0	1.18	3.11
	CK19: Low Pos	100	27.96	0.30	1.06	0.25	0.90	0 [a]	0	0.25	0.88	0.74	2.63
D (n=72)	MG: High Pos	100	17.66	0.56	3.19	0.16	0.91	0.37	2.11	0.42	2.36	0.61	3.47
	CK19: High Pos	100	20.03	1.37	6.82	0.95	4.76	0.28	1.38	0.83	4.12	0.57	2.85

a. According to NCCLS (CLSI) guideline EP05-A2, variance components less than 0 are recorded as 0.

CLINICAL PERFORMANCE

Two prospective, multi-site, U.S. clinical trials were conducted to gather the data from which to determine the proper Ct cutoffs for the GeneSearch™ BLN Assay (training set, Cutoff Study, 12 sites, n = 306) and to validate the chosen cutoffs in an independent subject set (test set, Pivotal Study, 11 sites, n = 423). Both studies had identical methods (described below).

Methods

Clinical site personnel performed the GeneSearch™ BLN Assay testing on SLNs freshly removed from female or male patients at least 18 years of age diagnosed with invasive breast cancer. The GeneSearch™ BLN Assay results were compared to rigorous permanent section H&E and IHC sectioning (described below) evaluated by pathologists who were blinded to assay results. Two independent pathologists evaluating a lymph node as having a metastatic focus > 0.2 mm were required for a lymph node (or subject) to be categorized as "positive." Results from the GeneSearch™ BLN assay were not used to make patient management decisions in the Pivotal Study.

Sentinel Lymph Node Cut-In and Sharing

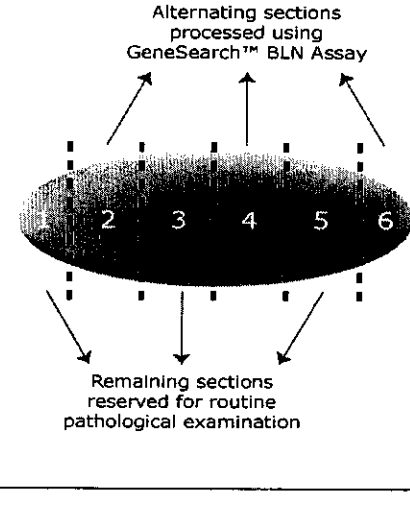
All sentinel lymph nodes were bisected along the short axis. Lymph nodes 6.0 mm or less in length were bisected to produce 2 lymph node tissue portions. Larger lymph nodes were cut along the short axis into an even number of lymph node tissue portions of approximately the same thickness, as indicated in Table. This procedure assured that all lymph node portions were between 1.5 to 3.0 mm in thickness, and that there were an equal number of tissue portions for histology and the GeneSearch™ BLN Assay. The figure shows an example of lymph node sharing between the GeneSearch™ BLN Assay and histology for a lymph node that is approximately 12 mm in length.

Table 9. Sentinel Lymph Node Cut-in and Sharing Between the GeneSearch™ BLN Assay and Histology as done in the Clinical Studies.

1.) Lymph node cut-in based on node size.
2.) An example of lymph node sharing between the assay and histology for a lymph node that is approximately 12 mm in length.

1. Lymph node cut-in based on node size	
Node size (longest dimension in mm)	**Total number of node tissue portions**
≤ 6	2
> 6 and ≤ 10	4
> 10 and ≤ 15	6
> 15 and ≤ 20	8
> 20	10 or more (each portion ≤ 3 mm thick)

2. Example of lymph node sharing



After any desired intra-operative touch preparation cytology (TPC) slides had been taken, alternating lymph node tissue portions from the single lymph node were combined, processed, and tested following the Sample Preparation, RNA Purification, and RT-PCR sections of this document.

Remaining tissue portions not used for the GeneSearch™ BLN Assay were processed for permanent section H&E for patient management using standard site procedures (Site Slides). Additional slides were also prepared from the fixed tissue for shipment to the study Central Pathologists for H&E evaluations (Central Slides). Central Slide sections were 4 to 6 μm thick, and 3 sections were taken from each 1.5 mm to 3.0 mm fixed lymph node portion. The 3 sections were taken from levels approximately 150 μm apart. Each site determined the number and levels of H&E sections to be evaluated by the site for patient management. IHC evaluations were done when H&E sections were found negative. At the site's discretion, these IHC slides may have been stained (as per standard site procedure) and evaluated by the Site Pathologists (Site Slides) or read by only the Central Pathologists (Central Slides). Thus, for each subject there were two separate sets of H&E slides (Site and Central) and for H&E negative subjects, one set of IHC slides (Site **or** Central).

Test Comparator - Histology Interpretation

The combination of permanent section H&E and IHC was used as the comparator test method in these studies to determine the performance of the GeneSearch™ BLN Assay. In an attempt to minimize some of the limitations of current histology, an "Overall Histology" result took into account all permanent section H&E and IHC results obtained on the subjects' lymph nodes, as described here. Two Central Pathologists independently read Central Slides. If the results from the two pathologists were discordant, a third Central Pathologist independently read the same slides. The final Central Slide result was determined by majority rule. For Site Slide results, if the site pathologist found the lymph node positive, whereas the Central Slides for the same lymph node were evaluated as negative, the Site Slides were sent to the Central Pathologists for confirmation of positivity. The lymph node (and patient) was considered histology positive if either Site Slides or Central Slides were confirmed positive. For either Site Slide or Central Slide evaluation, if only two pathologists' results were available with one being positive and one negative, the final result was considered undetermined (UND).

For assay performance calculations, histological results were divided into the following six categories in order of increasing levels of positivity:

N – negative, no evidence of tumor cells;

N(ITC) - isolated tumor cells only;

N(CL) - tumor cell clusters ≤ 0.2 mm;

P(MI) - micrometastasis > 0.2 to 2 mm;

P - metastasis > 0.2 mm but unknown specific size

P(MA) - macrometastasis > 2.0 mm

The first three categories were considered negative from a clinical perspective; the last three were considered positive. A lymph node was considered P(MA) only if two Central Pathologists agreed on that level of metastasis.

Central Slide H&E results were a requirement for inclusion of the lymph node in the study. When either the final Central Slide and/or final Site Slide histology result was positive, the Overall Histology result for the lymph node was "Positive." When Site Slide H&E results

and/or when any IHC results were not available, the existing Central slide histology result was the final histology result for the lymph node. If one set of slides had a final result of negative for a lymph node, and the other was UND, the final Overall Histology result for that lymph node was considered UND. For P(MA), P(MI), N(CL), N(ITC), or N, the Overall Histology result was always the more positive of the final Central or final Site Slide result.

Overall Histology Results Interpretation for a Subject

The subject's Overall Histology result was used for performance calculations for the GeneSearch™ BLN Assay. The subject's Overall Histology result was Negative if the Overall Histology result of all lymph nodes was negative, and Positive if at least one lymph node was positive. The subject's Overall Histology result was considered UND if one lymph node was evaluated as UND and there were no other lymph nodes, or all other lymph nodes were either UND or Negative. The subject's Overall Histology result was equal to the most positive result seen in any of their lymph nodes, e.g., if one lymph node was P(MA), one P(MI), and one negative, the patient result was P(MA).

Intra-operative Histological Evaluations

The sites' frozen section (FS) or intra-operative TPC results were collected to compare the performance of these intra-operative methods to the performance of the GeneSearch™ BLN Assay when each was measured against Overall Histology results as described above. There were no Central Pathologist readings of the intra-operative histology slides. The clinical trial sites collected intra-operative results for patient management either on a rare basis on special request from a given surgeon, or as standard practice for all patients undergoing SLND. For the GeneSearch™ BLN Assay clinical studies, sites were instructed to collect intra-operative histology as per their standard procedures, with the exception that 1) the lymph nodes were to be cut as indicated in Table, and 2) lymph node portions used for the investigational assay were not frozen. Frozen sections were taken only from the lymph node portions being used for histology. Intra-operative touch preparations could be taken from any lymph node portion, including those to be tested in the GeneSearch™ BLN Assay. Results from the GeneSearch™ BLN assay procedure were not used to make intra-operative surgical or therapy management decisions during the Pivotal Study.

Cutoff Study Results

Data from 274 subjects with valid GeneSearch™ BLN Assay results and defined Overall Histology were used to determine the cutoffs for the assay IC (PBGD), MG, and CK19 gene expression levels, i.e., cycle threshold values. Data from an additional 30 subjects with invalid GeneSearch™ BLN Assay results and 2 with Overall Histology results of UND were excluded from the cutoff determination, as these subjects lacked the definitive assay or histological determination of positive or negative needed to contribute to cutoff decisions. Cutoffs for PBGD, MG, and CK19 markers were determined as < 36 Ct, ≤ 31 Ct, and ≤ 30 Ct, respectively. Cutoffs were chosen to achieve a minimum specificity of 95% compared with the histological result. The performance of the GeneSearch™ BLN Assay with these cutoffs in the 274-subject data set was 91.1% sensitivity (95% confidence interval: 82.5% - 96.4%) and 95.9% specificity (95% confidence interval: 92.1% - 98.2%).

Pivotal Study Results

Subject Population

SLNs were removed during sentinel lymph node biopsy surgery from 423 subjects (418 females, 5 males) at least 18 years of age diagnosed with invasive breast cancer. Subject age ranged from 27 to 92 years with a mean age of 60. Nine subjects had chemotherapy and one had radiation therapy. The majority of subjects (80.4%) were diagnosed with invasive ductal cancer either alone or in combination with other breast cancer types. There were 13.9% of subjects with invasive lobular cancer but no invasive ductal cancer, and 5.7% with invasive cancer other than lobular or ductal. The majority of the subjects had either Stage I (62.3%) or Stage II (32.0%) breast tumors. Of the remaining subjects, 5.3% had Stage III tumors and 0.5% had Stage IV tumors. Mean size of the primary tumor was 1.9 centimeters. Subjects who had histologically detectable metastases in lymph nodes had larger primary tumors than subjects who had histologically undetectable metastases in lymph nodes. Most subjects were estrogen receptor (ER) positive (79.2%), progesterone receptor (PR) positive (67.8%), and HER-2 negative (74.2%).

SLN Disposition

The mean, median, and maximum numbers of lymph nodes removed were 2.9, 2, and 11, respectively. Two of the 423 subjects had no assay or study histology data available since their lymph nodes were too small to share tissue for study purposes.

Overall Histology Results

Five of the 421 subjects with assay results had indeterminate (UND) Overall Histology results and were not included in assay performance calculations. The prevalence of metastatic breast cancer in lymph nodes by Overall Histology for the remaining 416 subjects was 29.1% (121 positive subjects of 416), and ranged from 14.3 – 45.5% across all clinical sites. Metastatic breast cancer was found by H&E stain in 120 subjects. One subject identified with micrometastatic cancer, P(MI), was identified by IHC alone. Cancer metastases were most often P(MA) (77.7%), while 19.0% of subjects with metastases were categorized as P(MI), and 3.3% of undetermined size > 0.2 mm (P). Most subjects without H&E detectable metastases were completely histologically negative with no evidence of tumor cells (93.2% categorized as N), 4.7% of subjects were categorized as N(ITC), and 2.0% of subjects were categorized as N(CL).

GeneSearch™ BLN Assay Performance Calculations

The assay resulted in an invalid result for 34 (8.1%) of the 421 subjects, whether due to external control (13) or sample (21) failures. Subject invalid result rates were 4.2% when at least 40 assay runs had been completed by an operator, likely due to increased operator experience. These invalid results were not excluded in performance calculations, but were treated as assay "negative," since they do not provide the clinician with evidence of nodal metastases. Performance calculations were based on the 416 subjects with defined Overall Histology results (the five subjects with Overall Histology results of UND were not included). The GeneSearch™ BLN Assay overall performance (with 95% confidence intervals) is shown below with reference to Overall Histology. Also shown is assay

performance from the Cutoff Study evaluated by the same methods (assay invalids are treated as "negative" and subjects with incomplete histology are excluded).

Table 10. The GeneSearch™ BLN Assay Performance Characteristics Compared to Overall Histology.

STUDY	N	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Pivotal	416	87.6 (80.4-92.9)	94.2 (90.9-96.6)	86.2 (78.8-91.7)	94.9 (91.7-97.1)
Cutoff	304	82.4 (72.6-89.8)	96.3 (92.9-98.4)	89.7 (80.8-95.5)	93.4 (89.3-96.2)

PPV = Positive Predictive Value; NPV = Negative Predictive Value; CI= Confidence Interval

GeneSearch Result	Histology Positive	Histology Negative	Total
Positive	106	17	123
Negative	15	278	293
Total	121	295	**416**
Prevalence	29.1% (24.8-33.7)		

When only subjects with valid assay results and Overall Histology are considered, the following table represents the subject counts and overall performance. On a patient basis, there were 32 of 416 (7.7%) with discordant results between Overall Histology and the assay. Fourteen of 15 subjects with assay False Negative (FN) results had only one positive lymph node by Overall Histology. Of the 15 FN subjects, two (2) were due to external control failures being interpreted as "negative" for the purposes of assay performance calculations. In these two cases, subject sample Ct values were actually positive for both CK19 and MG. Thirteen of 15 FN subjects had valid assays. Most had small metastases with 9 being P(MI), 3 P, and only 1 P(MA) by Overall Histology. Eight of the 13 had positivity found only on Central Slides or Site Slides, but not on both.

There were a total of 17 subjects with assay False Positive (FP) results. Fifteen of the 17 were positive by the assay on only one lymph node.

Table 11. The GeneSearch™ BLN Assay Performance Characteristics compared to Overall Histology among Subjects with Valid Assay Results

STUDY	N	Sensitivity (95% CI)	Specificity (95% CI)	PPV ((95% CI)	NPV (95% CI)
Pivotal	383	90.6% (83.8-95.2)	93.6% (90.0-96.2)	86.2% (78.8-91.7)	95.8% (92.6-97.9)
Cutoff	274	91.9 (82.5-96.4)	95.9 (92.1-98.2)	90.0 (81.2-95.6)	96.4 (92.7-98.5)

GeneSearch Result	Histology Positive	Histology Negative	Total
Positive	106	17	123
Negative	11	249	260
Total	117	266	**383**
Prevalence	30.6% (26.0-35.4)		

The overall agreement between the GeneSearch™ BLN Assay and thorough permanent section histology was 92.7%; 95% confidence interval 89.6% to 95.1%.

The differences in results between the GeneSearch™ BLN Assay and Overall Histology could be due to tissue sampling since the assay evaluated different portions of the lymph node than did histology. This explanation is supported by differences between the histological evaluation of the two sets of H&E slides collected; a comparison of site pathologist results on Site H&E Slides to the central pathologist results on Central H&E Slides is described below. This is a comparison of H&E evaluations of anatomically close but different sections from the same portions of the lymph node.

Central Pathologist Agreement Evaluating the Same Slides

The overall positive/negative agreement on a subject level between the two primary central pathologists evaluating the same slides was 98.3%. There were 92 subjects evaluated with a macrometastasis by one or both central pathologists. Only 1 of these 92 subjects (1.1%) was found negative by the other pathologist. In seven of the 92 (7.6%) subjects, the other pathologist evaluated the subject as having micrometastases. There were 19 subjects evaluated by one or both central pathologists as having a micrometastasis (and not evaluated with macrometastases). In 31.6% of these cases (6/19), the other pathologist evaluated the subject as negative. These results illustrate the inherent difficulties of distinguishing between positivity and negativity for cancer metastases by histology.[5,6]

Agreement between Site Pathologist Results and Final Central Pathologist Results from Different H&E Slides

The site pathologist H&E results on Site Slides were compared with the central pathologist final H&E results on Central Slides among subjects with valid GeneSearch results. The overall agreement between these two evaluations was 92.2%. In seven cases one evaluation determined that the subject had macrometastases while the other evaluation found the subject

negative. In ten cases one evaluation determined that the subject had micrometastases while the other found the subject negative. Site pathology results were unavailable for 13 subjects. The specific comparisons are shown in Table 12 below:

Table 12: Agreement between Site Pathologist Results and Final Central Pathologist Results from Different H&E Slides

	Central Pathology				
Site Pathology	> 2 mm	0.2-2 mm	<0.2 mm	Negative	Total
>2 mm	79	3	0	4	86
0.2-2 mm	5	9	0	9	23
<0.2 mm	0	2	0	4	6
Negative	3	1	1	288	293
#N/A	2	3	0	8	13
Total	89	18	1	313	421

The lack of perfect agreement could be due to different sections (samplings) from the lymph node tissue being taken for Site Pathology evaluation versus Central Pathology evaluation, since significant metastases can be missed in nearby tissue left uncut on the block.[7, 8] It is reported that the non-uniform distribution of micrometastases in lymph nodes lowers the probability of correctly evaluating metastases at earlier stages of breast cancer.[7, 5]

Confirmation of Site Slide Positivity

There were 109 subjects reported positive by the site pathologists among 421 subjects. Central Slides were also positive in 88.1% of those cases (96 0f 109). Of the remaining 13 subjects reported positive by the site and found negative on Central Slides, central pathologist(s) confirmed positivity on the Site Slides in 61.5% (8/13). In 30.8% (4/13), both central pathologists found the Site Slides negative. In one additional site-positive subject, missing third central pathologist data precluded confirmation of Site Slide positivity. These data illustrate the limitations of sampling and histological evaluation.[9]

Assay Performance within Patient Subsets

Although the GeneSearch™ BLN Assay is not designed to distinguish between micrometastatic and macrometastatic disease in lymph nodes, summary statistics were calculated for positive and negative assay results across the spectrum of histologic findings. The calculations were based on 380 subjects from the 423 enrolled in the pivotal study, omitting subjects with insufficient lymph node tissue for study, subjects whose Overall Histology results were either UND or Positive without specifying micro- vs macrometastatic disease, and subjects with invalid assay results. The GeneSearch™ BLN assay was positive for 92 of the 93 subjects with P(MA) by Overall Histology (98.9%; 95% Confidence Interval: 94.1 - 99.9%). It was negative for 257 of the 287 subjects whose Overall Histology was Negative or P(MI) (89.5%; 95% Confidence Interval: 85.4 - 92.8%). The GeneSearch™ BLN Assay was positive for 13 of 21 P(MI) subjects (61.9%; 95% Confidence Interval: 38.4 - 81.9%), and negative for 249 of 266 subjects with Negative Overall Histology (93.6%; 95% Confidence Interval: 90.0 - 96.2%). The results on P(MI) subjects show the difficulty of

detecting small and infrequently occurring metastases, perhaps due to limited sampling[9]. Note that the sensitivity for micrometastases for Site Pathology H&E compared to Central Pathology H&E on different, but nearby sections of the lymph node, was 80.0% (95% Confidence Interval: 51.9 - 95.7%).

There were 31 subjects who had breast surgery conducted immediately prior to the SLND. There was 100% agreement between the Overall Histology result and the GeneSearch™ BLN Assay result in these subjects, suggesting that conducting breast surgery prior to the SLND did not cause false positive assay results due to contamination of the SLNs with breast tissue.

For the five male subjects, assay results were in agreement with Overall Histology. Three were Negative (N) and two were P(MA).

There were 10 subjects who were receiving cancer treatment by chemotherapy (9) or radiation (1). The assay results agreed with Overall Histology (7 True Negative (TN) and 2 True Positive (TP)) in all but one of these subjects. That subject was receiving chemotherapy and was negative by the GeneSearch™ BLN Assay and H&E, and P(MI) only by IHC. The GeneSearch™ BLN Assay, Site Slides H&E and Central Slides H&E were negative for this subject.

Table 13 shows the performance of the GeneSearch™ BLN Assay by tumor histology and tumor size.

Table 13. GeneSearch™ BLN Assay Sensitivity and Specificity Relative to Final Histology Results by Tumor Histology and Tumor Size

	N	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Tumor Histology					
Invasive Ductal	335	88.8 (80.8-94.3)	94.1 (90.3-96.7)	86.1	95.3
Invasive Lobular	57	80.0 (56.3-94.3)	91.9 (78.1-98.3)	84.2	89.5
Invasive Other	24	100 (29.2-100)	100 (83.9-100)	100	100
Tumor Size [a]					
< 1 cm	88	90.9 (58.7-99.8)	100 (95.3-100)	100	98.7
1 - < 2 cm	159	81.4 (66.6-91.6)	92.2 (85.8-96.4)	79.5	93.0
2 – 4.9 cm	127	90.9 (78.3-97.5)	90 (81.2-95.6)	83.3	94.7
≥ 5 cm	22	92.3 (64.0-99.8)	100 (63.1-100)	100	88.9

a. Tumor size was not available for 25 subjects.

The number of lymph nodes found positive in a subject by the GeneSearch™ BLN Assay compared to the number found positive in the same subject by Overall Histology is shown in

detecting small and infrequently occurring metastases, perhaps due to limited sampling[9]. Note that the sensitivity for micrometastases for Site Pathology H&E compared to Central Pathology H&E on different, but nearby sections of the lymph node, was 80.0% (95% Confidence Interval: 51.9 - 95.7%).

There were 31 subjects who had breast surgery conducted immediately prior to the SLND. There was 100% agreement between the Overall Histology result and the GeneSearch™ BLN Assay result in these subjects, suggesting that conducting breast surgery prior to the SLND did not cause false positive assay results due to contamination of the SLNs with breast tissue.

For the five male subjects, assay results were in agreement with Overall Histology. Three were Negative (N) and two were P(MA).

There were 10 subjects who were receiving cancer treatment by chemotherapy (9) or radiation (1). The assay results agreed with Overall Histology (7 True Negative (TN) and 2 True Positive (TP)) in all but one of these subjects. That subject was receiving chemotherapy and was negative by the GeneSearch™ BLN Assay and H&E, and P(MI) only by IHC. The GeneSearch™ BLN Assay, Site Slides H&E and Central Slides H&E were negative for this subject.

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Invasive Other	24	100 (29.2-100)	100 (83.9-100)	100	100
Tumor Size [a]					
< 1 cm	88	90.9 (58.7-99.8)	100 (95.3-100)	100	98.7
1 - < 2 cm	159	81.4 (66.6-91.6)	92.2 (85.8-96.4)	79.5	93.0
2 – 4.9 cm	127	90.9 (78.3-97.5)	90 (81.2-95.6)	83.3	94.7
≥ 5 cm	22	92.3 (64.0-99.8)	100 (63.1-100)	100	88.9

a. Tumor size was not available for 25 subjects.

The number of lymph nodes found positive in a subject by the GeneSearch™ BLN Assay compared to the number found positive in the same subject by Overall Histology is shown in

Table 14 below. The Kappa value[10] of agreement between the two tests was 0.75 (95% Confidence Interval: 0.68 to 0.81).

Table 14. Number of lymph nodes found positive in the same subject by Overall Histology

Histology	GeneSearch™ BLN Assay (No. Positive Nodes)					
(No. Positive Nodes)	**0 (NEG)**	**1**	**2**	**3**	**≥4**	**Total**
0 (NEG)	278	15	1	1	0	295
1	14	57	8	1	0	80
2	1	5	23	1	0	30
3	0	0	1	3	2	6
≥4	0	0	0	1	4	5
Total	293	77	33	7	6	416

Shaded cells indicate 100 % agreement.

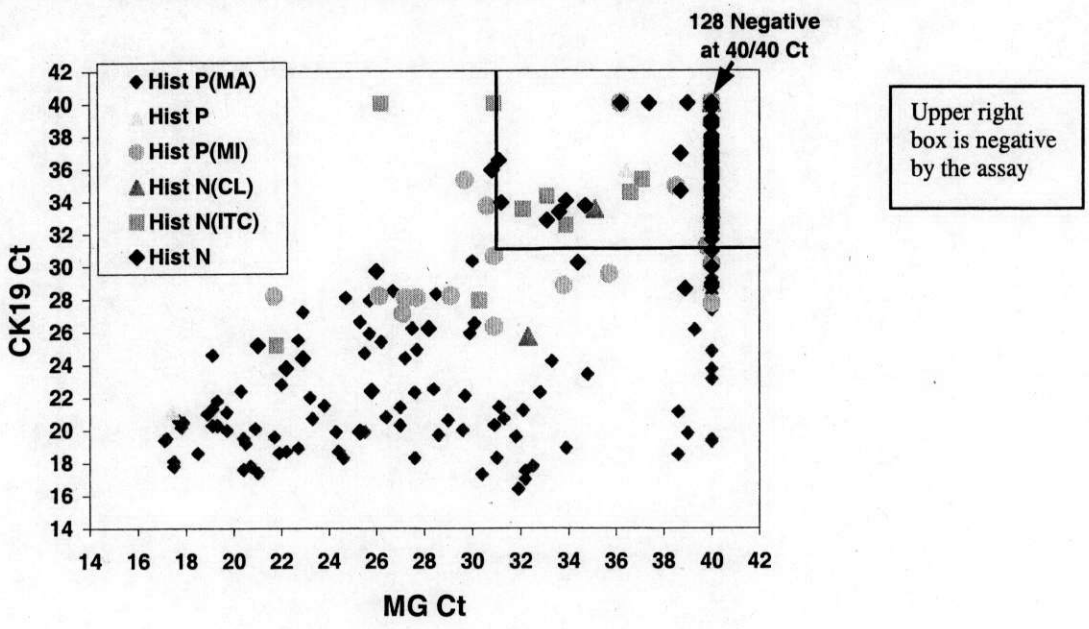
Of the 17 cases where the assay was interpreted as falsely positive (assay positive in 1 or more nodes but histology negative), 15 (88.2%) subjects were identified as assay positive in a single lymph node. Of 295 subjects who were characterized as Negative by Overall Histology, these 17 subjects (5.8%) would have been categorized as positive by the assay. Of 121 subjects who were categorized as positive by Overall Histology, 15 subjects (12.4%) would have been categorized as negative by the assay. Of these 15 cases, 14 subjects were identified as histologically positive in a single lymph node. In summary, for node status, the assay categorized a total of 32 patients (7.7%) differently than did Overall Histology. Concordance between the assay and histology for the number of positive lymph nodes per patient between histology and assay is imperfect. The reasons for divergence are not established.

Staging is a histological exercise and the GeneSearch™ BLN Assay is not intended for staging purposes. The 2002 AJCC revised staging system[11] for breast cancer includes mention of molecular techniques such as RT-PCR used to detect nodal metastases. According to that system, and absent histologic findings of sentinel lymph node metastases, positive results from a molecular test are consistent with a staging status of pN0(mol+). Test results are not otherwise intended for use in assigning tumor stage.

Details of Ct Values and Overall Histology in the Pivotal Study

Correlations were observed between assay cancer analyte Ct values and level of metastases reported by Overall Histology (n = 383, conclusive histology and valid GeneSearch™ BLN Assay results). The rank correlation coefficient was 0.77 for MG and 0.74 for CK19 Ct values *versus* the six histology categories of P(MA), P, P(MI), N(CL), N(ITC) and N which were ranked 1-6 for the analysis. The following graph shows that relationship. There are 128 overlapping subjects with negative histology at the 40/40 point in the graph. Those subjects with CK19 Ct values less than 25 or MG Ct values less than 26 were more likely to have macro-metastases (80/88, 90.9%).

Figure 3. Spearman Nonparametric Correlation Coefficient Analysis.



Comparison of the GeneSearch™ BLN Assay Performance to Current Intra-operative Tests

The performance of the GeneSearch™ BLN Assay was compared to that of current intra-operative methods that were in use at the Pivotal Study sites. For comparison with intra-operative FS, the data set was limited to those 319 subjects who had both intra-operative FS and BLN assay results and who had a conclusive Overall Histology result. Frozen section was performed according to locally determined indications and techniques. Intra-operative TPC results were available on only 29 subjects, but are also reported for comparison. In all cases, the reference test was Overall Histology. For the purposes of performance calculations, BLN assay invalid results were included and treated as assay "negative," since these results do not provide the clinician with evidence of nodal metastases.

Table 15. Comparison of the GeneSearch™ BLN Assay performance to that of other intra-operative methods used in Pivotal Study sites.

Test	N	Sensitivity (95% Confidence Interval)	Specificity (95% Confidence Interval)	PPV	NPV
BLN Assay	319*	95.6 (89.0-98.8)	94.3 (90.5-96.9)	86.9	98.2
FS	319*	85.6 (76.6-92.1)	97.8 (95.0-99.3)	93.9	94.5
TPC	29	45.5 (16.7-76.6)	100 (81.5-100)	100	75.0

*BLN Assay and FS were compared on subjects for whom both results were available

Sensitivity of the GeneSearch™ BLN Assay was 95.6% compared to 85.6% for FS and 45.5% for TPC. Sensitivity of the GeneSearch™ BLN Assay for subjects with P(MA) was 100% (76/76) compared to 90.8% (69/76) for FS and 57.1% (4/7) for TPC. Sensitivity of the GeneSearch™ BLN Assay for subjects with P(MI) was 81.8% (9/11), and was 54.5% (6/11) for FS and 25.0% (1/4) for TPC.

Specificity for FS was 97.8% and for TPC was 100% while the specificity for the GeneSearch™ BLN assay was 94.3%. Due to the limited sampling involved in current intra-operative techniques, adjacent and more thorough permanent section histology is likely to confirm metastases seen with FS or TPC. The specificity of FS diagnosis is commonly reported as near 100%, perhaps due in part to the ability to re-examine the exact tissue used to make original FS diagnoses. FS re-examination was not part of the pivotal study, since an analogous histologic re-examination of the exact tissue for the GeneSearch™ BLN Assay is not possible.

When comparing FS with the BLN assay using Overall Histology as the standard, the following specific results were obtained:

Overall Histology Positive			
	Frozen section result		
GeneSearch™ result	Positive	Negative	Total
Positive	75	11	86
Negative	2	2	4
Total	77	13	90

Overall Histology Negative			
	Frozen section result		
GeneSearch™ result	Positive	Negative	Total
Positive	0	13	13
Negative	5	211	216
Total	5	224	229

The GeneSearch™ BLN assay found 86 out of 90 histology Positives while FS found 77 of 90 histology Positives. In 229 histology negative subjects, 13 were false positives by the GeneSearch™ BLN Assay and 5 were false positives by FS.

These data show that the GeneSearch™ BLN Assay detected more metastases than did current frozen section, despite the fact that the comparator test (Overall Histology) was conducted on different portions of the lymph node than those on which the assay was conducted. FS results were, in contrast, generated using sections obtained closer to the sections that were used for Overall Histology.

The performance of the GeneSearch™ BLN Assay in combination with other intra-operative histological techniques has not been examined.

Discussion of Performance Results

As done for any diagnostic test used to make intra-operative decisions, when a patient is scheduled to undergo sentinel lymph node biopsy and the assay will be used intra-operatively to contribute to a decision to proceed or not to further lymph node dissection, the patient should be made aware of the risks and benefits in the use of the assay. The assay risks and benefits should be discussed with the patient prior to surgery. The information should be based upon the above clinical studies and the consequences of accurate and inaccurate diagnoses of metastatic cancer. The following information can contribute to this discussion with the patient.

The likelihood of an assay positive result when Overall Histology is Positive (> 0.2 mm) is 87.6% (see Table 10), at worst 80.4% (lower 95% confidence interval), at best 92.9% (upper 95% confidence interval). An assay result used intra-operatively that was false negative compared to later positive permanent section histology results would mean that any necessary additional axillary lymph node dissection would be performed in a second surgery rather than during the surgery for sentinel lymph node biopsy. In histologically positive patients, the likelihood of an assay false negative result and the failure to be able to perform the additional axillary node dissection during the same surgery is 12.4%, at worst 19.6%, at best 7.1%.

The likelihood of an assay negative result when Overall Histology is Negative is 94.2%, at worst 90.9%, at best 96.6%. An assay result used intra-operatively that was false positive compared to later negative permanent section histology results would mean that a complete axillary dissection performed on the basis of the positive assay result during the same surgery as the sentient lymph node biopsy procedure may have been unnecessary based on histological evidence alone. In histologically negative patients, the likelihood of an assay false positive result and the possibility of an unnecessary complete axillary node dissection is 5.8%, at worst 9.1%, at best 3.4%.

The total rate of disagreement that can be expected between the assay and permanent section histology as performed in the Pivotal Study is 7.7% (at worst 10.7%, at best 5.3%). This represents 32 subjects of 416 in the Pivotal Study with disagreement between the assay and histology results -- 15 assay false negatives and 17 assay false positives.

When a patient tests assay negative, there is a 94.9% likelihood (at worst 91.7%, at best 97.1%) that later histology will also be negative. When a patient tests assay negative and histologically detectable metastatic cancer is found later, that patient will typically require a second surgical procedure for complete axillary node dissection. In assay negative patients, the likelihood of histologically detectable metastatic cancer is 5.1%; at worst 8.3%, at best 2.9%.

When a patient tests assay positive, the chance histologically detectable cancer also will be found is 86.2% (see Table 9), at worst 78.8%, at best 91.7%. When a patient tests assay positive and histologically detectable metastatic cancer is not found later, a complete axillary dissection may have been performed unnecessarily on the basis of the assay positive result. In assay positive patients, the likelihood that histologically detectable metastatic cancer will not be found is 13.8%, at worst 21.2%, at best 8.3%.

In addition to the potential morbidity from the axillary lymph node dissection, there is a potential for additional morbidity due to over-treatment with adjuvant therapy. If any

axillary nodes show histologically detectable cancer, then the axillary dissection was likely warranted despite the sentinel nodes testing histologically negative. The likelihood of the presence of histologically detectable metastatic cancer in axillary nodes when sentinel nodes are histologically negative is approximately 5-10%[12-14]

Patients with false negative assay results may not receive a needed axillary node dissection and potentially fail to receive needed adjuvant therapy or would incur a delay in such therapy. Failure to adequately treat metastatic cancer can result in earlier distant metastases and increased risk of shorter long-term survival [15-17]. A falsely negative assay result may be recognized when the residual node portions are evaluated within days by permanent section histology. Such patients could then have an appropriate axillary node dissection in a second surgery and likely would receive the appropriate adjuvant therapy. Permanent section histology itself conducted following current guidelines[18] is reported to have false negative rates of 9-12.7%[19,20].

A summary of potential consequences of true and false assay test results (compared to permanent section histology) is as follows:

True Positive	**False Positive**
• ALND performed in same surgery, avoiding stress, costs, and risks associated with a second surgery • Histologically verified as positive on permanent sections from same nodes • Potential clinical benefit from excising and identifying metastatic nodes	• ALND performed on patient with sentinel nodes lacking histologically identified metastases • Potential for having performed an unnecessary ALND. If positive nodes found from ALND, ALND was likely of benefit to the patient. (Note: in patients with SLNDs found positive by histology, approximately 52-58%[14,21] will not have histologically identified metastases in the nodes removed during the ALND) • Potential over-treatment and its associated increased morbidity • Little or no clinical benefit from excising histologically negative nodes
True Negative	**False Negative**
• No ALND performed • Histologically verified as negative on permanent sections from the same nodes • More of the node examined than would have been done by histology alone giving more confidence in an accurate negative result and appropriate treatment decisions for the patient	• Necessary ALND not performed during sentinel lymph node surgery, patient recalled for second operation. Increased stress, costs, and risks associated with a second surgery for ALND • Permanent section histology detects nodal metastases in sentinel nodes 1-3 days after sentinel lymph node procedure

Summary of Clinical Performance

The agreement between the GeneSearch™ BLN Assay and thorough permanent section histology (Overall Histology being site and central slides) with review by at least two independent pathologists (355 of 383 subjects with valid assay results) was 92.7%; 95% confidence interval 89.6% to 95.1% (Table 11). This is similar to the agreement between Site Pathology review versus Central Pathology review of different H&E sections from the same lymph nodes (389 of 408 = 95.3%; 95% confidence interval 90.8% to 97.2%). In the Pivotal Study, subject invalid result rates were 4.2% when at least 40 assay runs had been completed by an operator.

The sensitivity was 87.6% (see Table 10), at worst 80.4% (lower 95% confidence interval), at best 92.9% (upper 95% confidence interval). Therefore, the chance of an assay negative, or false negative, result when histology positive is 12.4%, at worst 19.6%, at best 7.1%. The specificity was 94.2%, at worst 90.9%, at best 96.6%. The chance of an assay positive, false positive, result when histology is negative is 5.8%, at worst 9.1%, at best 3.4%. Sensitivity of the GeneSearch™ BLN Assay for 94 subjects with histologically identified macrometastases was 97.9% (95% Confidence Interval: 92.5 – 99.7%). For 23 subjects with histologically identified micrometastases, assay sensitivity was 56.5% (95% Confidence Interval: 34.5-76.8%).

Of 295 subjects with histologically negative lymph nodes, 5.8% were categorized as node positive by the assay while 94.2% were categorized as node negative by the assay. Of 121 subjects with histologically positive lymph nodes, 12.4% were categorized as node negative by the assay while 87.6% were correctly characterized as node positive.

In a matched data set, the GeneSearch™ BLN Assay sensitivity was 95.6% compared to permanent section H&E and intra-operative frozen section sensitivity was 85.6% evaluation (86 of 90 for the assay *versus* 77 of 90 for frozen section evaluation). When permanent section histology was negative, the GeneSearch™ BLN assay identified 3.5% more patients as positive than did the frozen section evaluation (13 of 229 for the assay *versus* 5 of 229 for frozen section evaluation).

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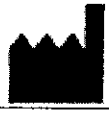	Manufacturer
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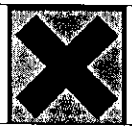	Harmful

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